High Sensitivity Direct Cyclic GMP

Chemiluminescent Immunoassay Kit

User Manual

Catalog # K3020-1 1 Plate Kit
K3020-5 5 Plate Kit

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INTENDED USE

The B-Bridge High Sensitivity Direct Cyclic GMP Chemiluminescent Immunoassay Kit is species independent and quantitatively measures cGMP in Cell Lysates, Saliva, Urine, EDTA Plasma and Tissue Culture Media. This kit can measure as little as 1 femtomol cGMP per sample. Please read the complete kit insert before performing this assay.

BACKGROUND

Guanosine 3', 5'-cyclic monophosphate (cyclic GMP; cGMP) is a critical and multifunctional second messenger present at levels typically 10-100 fold lower than cAMP in most tissues. Intracellular cGMP is formed by the action of the enzyme guanylate cyclase on GTP and degraded through phosphodiesterase hydrolysis. Guanylate cyclases (GC) are either soluble or membrane bound. Soluble GCs are nitric oxide responsive, whereas membrane bound GCs respond to hormones such as acetylcholine, insulin and oxytocin. Other chemicals like serotonin and histamine also cause an increase in cGMP levels. Cyclic GMP regulates cellular composition through cGMP-dependent kinase, cGMP-dependent ion channels or transporters, and through its hydrolytic degradation by phosphodiesterase.

ASSAY PRINCIPLE

The B-Bridge Direct High Sensitivity Cyclic GMP (cGMP) Chemiluminescent Immunoassay kit is designed to quantitatively measure cGMP present in Cell Lysates, Saliva, Urine, EDTA Plasma and Tissue Culture Media.

For samples where the levels of cGMP are expected to be relatively high, the regular format for the assay can be used. For samples with expected low levels of cGMP, an optional acetylation protocol can be used.

The kit is unique in that all samples and standards are diluted into an acidic Sample Diluent, which contains special additives and stabilizers, for cGMP measurement. This allows plasma, urine and saliva samples to be read in an identical manner to lysed cells. Acidified samples of cGMP are stable and endogenous phosphodiesterases are inactivated in the Sample Diluent.

1. Plate primer added to microtiter plate wells followed by sample or standard.
2. The reaction is initiated with the addition of the cGMP-peroxidase conjugate and chemiluminescent substrate.
3. Incubate overnight and use plate reader to ascertain luminescence.
4. Calculate cGMP concentration from standard curve.

KIT COMPONENTS

Component:	Cat #	K3020-1	K3020-5
Coated white 96-well plate		1 plate	5 plates
Cyclic GMP Standard (640 pmol/mL)		70 µl	70 µl
Cyclic GMP CLIA Antibody		3 ml	13 ml
Cyclic AMP Conjugate CLIA Concentrate		150 µl	650 µl
Conjugate Diluent		3 ml	13 ml
Sample Diluent Concentrate - **WARNING: Caustic**		12 ml	60 ml
Plate Primer		25 ml	25 ml
Acetic Anhydride - **WARNING: Corrosive Lachrymator**		2 ml	2 ml
Triethylamine - **WARNING: Corrosive Lachrymator**		4 ml	4 ml
20X Wash Buffer Concentrate		30 ml	125 ml
Substrate Solution A		6 ml	28 ml
Substrate Solution B		6 ml	28 ml
Plate Sealer		1 each	5 each

Store all components at 4°C

MATERIALS REQUIRED BUT NOT SUPPLIED

- Deionized or distilled water
- Microplate Shaker
- Glass test tubes
- 96-well plate reader capable of reading glow chemiluminescence
- Software for converting raw relative light unit (RLU) readings from the plate reader and carrying out four parameter logistic curve (4PLC) fitting

Note: Dilute 5 µL of the Cyclic GMP CLIA Conjugate Concentrate into 495 µL of deionized water. Pipet 5 µL of diluted conjugate into a white well and add 100 µL of prepared CLIA substrate. This well will give you an intensity slightly above the maximum binding for the assay. Adjust the gain or sensitivity so that your reader is giving close to the maximum signal.

PRECAUTIONS

This kit should only be used by qualified personnel who have had laboratory safety instruction. The complete User Manual should be read and understood before using this product.

Sodium azide in buffers will irreversibly inhibit the activity of peroxidase enzymes. Samples in any buffer containing azide will need to be diluted substantially, typically >1:100.

The supplied Sample Diluent and Sample Diluent Concentrate are acidic. Take appropriate precautions when handling these reagents. The kit uses acetic anhydride and triethylamine as acetylation reagents. Triethylamine and acetic anhydride are lachrymators. **Caution - corrosive, flammable, and harmful vapor. Use in hood with proper ventilation and wear appropriate protective safety wear.**

In all cases, please consult your institution's safety procedures for working with hazardous chemicals.

INTERFERENTS

A variety of detergents were tested as possible interfering substances in the assay. CHAPS, and Tween 20 at 0.1% increased measured cAMP by 8.9 and decreased measured cAMP by 0.9% respectively. Triton X-100 at 2% increased measured cAMP by 1.8% and CTAC at 0.05% increased measured cAMP by 6.3%. Samples containing SDS above 0.01% should not be used in the assay.

SAMPLE PREPARATION

Samples can be used within 2 hours or preparation or stored long-term at -70°C. 24-Hour urine samples may need to have 1 mL concentrated hydrochloric acid added for every 100 mL volume to act as a preservative. Samples containing visible particulate should be centrifuged prior to using.

After dilution in the Sample Diluent (see page 9) there may be some precipitation of proteins and the supernatant from the centrifuged samples used. After being diluted in Sample Diluent the samples can be assayed directly within 2 hours, or frozen at ≤ -70°C for later analysis. Severely hemolyzed samples should not be used in this kit.

For samples containing low levels of cGMP, the acetylated assay protocol must be used due to its enhanced sensitivity. **All standards and samples should be diluted in glass test tubes.**

Cells
Cell lysis buffers containing high concentrations of SDS or other detergents may not be compatible with this assay or may require extra dilution. Please read Interferents section above for more information.
This kit is compatible with either adherent or non-adherent cells. The cells can be grown in any suitable sterile containers such as Petri dishes, 12-, 48- or 96-well culture plates or flasks. The cells must be isolated from the media prior to being lysed with the provided Sample Diluent. The acidic Sample Diluent contains detergents to lyse the cells, inactivate endogenous phosphodiesterases and stabilize the cGMP. Some cell types are extremely hardy and the end user should optimize the lysis conditions utilizing freeze-thaw cycles and ultrasonic treatments to fully lyse their cells.

For adherent cells, the media should be aspirated from the cells and the cells washed with PBS. The adherent cells should be treated directly with the Sample Diluent for 10 minutes at room temperature. Cells can be scraped to dislodge them from the plate surface and cells should be inspected to ensure lysis. Detergent has been added to the Sample Diluent to help lysis occur. Centrifuge the samples at ≥600 x g at 4°C for 15 minutes and assay the supernatant directly. If required, the TCM can be assayed for cGMP as outlined below.

For non-adherent cells, pellet and wash the cells with PBS by centrifuging the samples at ≥600 x g at 4°C for 15 minutes as described above. Treat the aspirated, washed pellet directly with the Sample Diluent for 10 minutes at room temperature. Cells should be inspected to ensure lysis. Detergent has been added to the Sample Diluent to help lysis occur. Centrifuge the samples at ≥600 x g at 4 °C for 15 minutes and assay the supernatant directly. If required, the TCM can be assayed for cGMP as outlined below.

Tissue Samples
Tissues samples should be frozen in liquid nitrogen and stored at -80°C if analysis is not to be carried out immediately.

Grind the frozen tissue in a stainless steel mortar under liquid nitrogen until it is a fine powder. Allow the liquid nitrogen to evaporate and weigh the powdered tissue. Add 1 mL of Sample Diluent for every 100 mg of tissue. Incubate in the Sample Diluent for 10 minutes on ice, and then centrifuge at ≥600 x g at 4°C for 15 minutes. Collect the supernatant and run in the assay immediately or store frozen at ≤ -70°C.

For samples that require concentration and delipidation, a trichloroacetic acid (TCA)/ether protocol can be used. Grind the frozen tissue in a stainless steel mortar under liquid nitrogen until it is a fine powder. Allow the liquid nitrogen to evaporate and weigh the powdered tissue. Add 1 mL of ice cold 5% TCA (weight/volume) for every 100 mg of tissue and grind in a glass-Teflon mortar. Incubate in the TCA for 10 minutes on ice, and then centrifuge at ≥600 x g at 4°C for 15 minutes. Collect the supernatant.

For every 1 mL of TCA supernatant add 3 mL of water saturated diethyl ether* and shake in a glass vial. Allow the ether to separate as the top layer, remove it and discard the ether. Dry the aqueous layer by lyophilization or using a vacuum centrifuge. Reconstitute by adding 1 mL of Sample Diluent for every mL of 5% TCA used to extract and run in the assay immediately or store at ≤ -70°C.
Diethyl ether is extremely flammable and should be used in a hood.

Tissue Culture Media
For measuring cGMP in tissue culture media (TCM), samples should be read off a standard curve generated in TCM. Samples may need to be diluted further in TCM. We have validated the assay using RPMI-1640.

Plasma Samples
Plasma samples should be diluted ≥ 1:10 with the supplied Sample Diluent and acetylated prior to running in the Acetylated Format assay.

Urine Samples
Urine samples should be diluted ≥ 1:20 with the supplied Sample Diluent prior running in the assay. Due to the high concentration of cGMP in urine, samples may need to be diluted further.

Saliva Samples
Saliva samples should be diluted ≥ 1:4 with the supplied Sample Diluent prior running in the assay.

Use all samples within 2 hours of dilution in Sample Diluent.

REGULAR FORMAT ASSAY

REGULAR FORMAT REAGENT PREPARATION

Allow the kit reagents to come to room temperature for 30-60 minutes.

We recommend that all standards and samples be run in duplicate to allow the end user to accurately determine cAMP concentrations. Ensure that all samples have reached room temperature and have been diluted as appropriate prior to running them in the kit.

Wash Buffer
Dilute Wash Buffer Concentrate 1:20 by adding one part of the concentrate to nineteen parts of deionized water. Once diluted this is stable at room temperature for 3 months.

Cyclic GMP Conjugate Concentrate
The supplied Cyclic GMP Conjugate Concentrate should be diluted 1:20 with the Conjugate Diluent (ie: 125uL Conjugate Concentrate with 2.375 mL of Conjugate Diluent for a total solution volume of 2.5 mL) Once diluted the Cyclic GMP conjugate is stable for one month when stored at 4°C.

Chemiluminescent Substrate

Mix one part of the Substrate Solution A with one part of Substrate Solution B in a brown bottle. Once mixed the substrate is stable for one month when stored at 4°C.

Standard Preparation
All standards and samples should be diluted in glass test tubes.

1. Label one glass test tube as Working Stock and seven tubes as #1 through #7.
2. Pipet 150 µL of Sample Diluent into the Working Stock tube and 296 µL of Sample Diluent into tube #1.
3. Pipet 150 µL of Sample Diluent into tubes #2 to #7.
4. The Cyclic GMP stock solution contains an organic solvent. Prerinse the pipet tip several times to ensure accurate delivery. Carefully add 10 µL of the cGMP stock solution to the Stock 2 tube and vortex completely.
5. Take 24 µL of the cGMP solution in the Working Stock tube and add it to tube #1 and vortex completely.
6. Take 150 µL of the cGMP solution in tube #1 and add it to tube #2 and vortex completely.
7. Repeat the serial dilutions for tubes #3 through #7. The concentration of Cyclic GMP in tubes 1 through 7 will be 3, 1.5, 0.75, 0.375, 0.188, 0.094, and 0.047pmol/mL.

Use Standards within 1 hour of preparation.

Reagent	Working Stock	Standard 1	Standard 2	Standard 3	Standard 4	Standard 5	Standard 6	Standard 7
Sample Diluent	150 µl	296 µl	150 µl	150 µl	150 µl	150 µl	150 µl	150 µl
cGMP Standard Stock Solution	10 µl							
Working Stock		24 µl						
Standard 1			150 µl					
Standard 2				150 µl				
Standard 3					150 µl			
Standard 4						150 µl		
Standard 5							150 µl	
Standard 6								150 µl
Final Concentration (pM/ml)	40	3	1.5	0.75	0.375	0.1875	0.0938	0.0469

REGULAR FORMAT ASSAY PROTOCOL

1. Add 50 µL of Plate Primer into all wells used.
   Failure To Add Plate Primer To ALL Wells First Will Cause Assay To Fail.

2. Pipet 75 µL Sample Diluent into the non-specific binding (NSB) wells.

3. Pipet 50 µL of Sample Diluent into wells to act as maximum binding wells (B0 or 0 pg/mL).

4. Pipet 50 µL of samples or standards into wells in the plate.

NOTE: Sample Diluent will turn from orange to bright pink upon sample or standard addition to the Plate Primer in the wells.

5. Add 25 µL of the diluted cGMP Conjugate to each well using a repeater or multichannel pipet.

6. Add 25 µL of the cAMP Antibody to each well, except the NSB wells, using a repeater or multichannel pipet.

7. Cover the plate with the plate sealer and shake the plate for 15 minutes at room temperature.

8. Place the covered plate in a 4°C refrigerator for 16 hours.

9. The next morning take the plate from the refrigerator and wash the plate 4 times with the wash buffer. Tap the plate dry on absorbent towels.

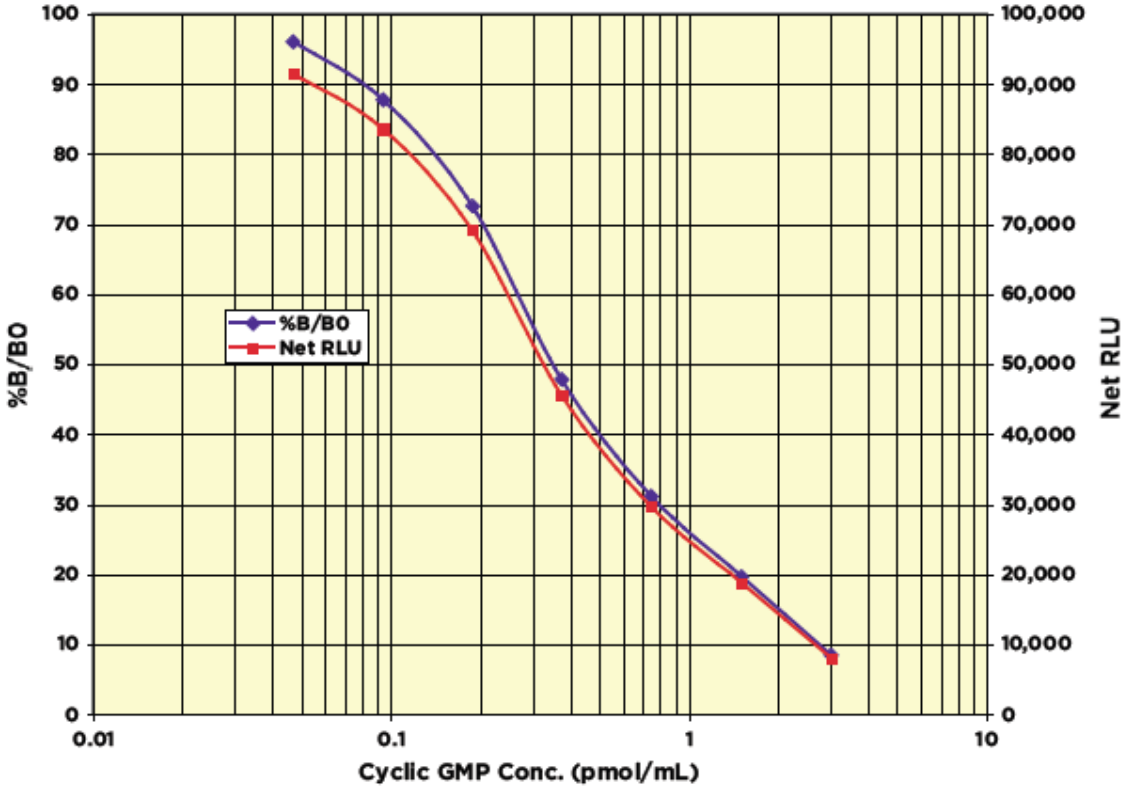
10. Add 100 µL of the mixed Chemiluminescent Substrate to each well, using a repeater or multichannel pipet.

11. Immediately read the luminescence generated from each well in a mutimode or chemiluminescent plate reader using a 0.1 second read time per well. The chemiluminescent signal will decrease about 40% over 60 minutes.

CALCULATIONS

All luminometers read Relative Light Units (RLU). These RLU readings will vary with make or model of plate reader. Average the duplicate RLU readings for each standard and sample. Create a standard curve by reducing the data using the 4PLC fitting routine on the plate reader, after subtracting the mean RLU's for the NSB. The sample concentrations obtained, calculated from the %B/B0 curve, should be multiplied by the dilution factor to obtain neat sample values.

REGULAR FORMAT TYPICAL STANDARD CURVE: EXAMPLE



VALIDATION DATA: REGULAR FORMAT

Sensitivity and Limit of Detection
Sensitivity was calculated by comparing the RLU's for twenty wells run for each of the B0 and standard #7. The sensitivity was determined at two (2) standard deviations from the B0 along the standard curve.
Sensitivity was determined as 0.034 pmol/mL.

The Limit of Detection for the assay was determined in a similar manner by comparing the RLU's for twenty runs for each of the zero standard and a low concentration human urine sample.
Limit of Detection was determined as 0.047 pmol/mL

ACETYLATED ASSAY

Use this format for any sample with low cGMP concentrations.

Prior to running the acetylated assay, all standards, samples and the Sample Diluent used for the B0 and NSB wells must be acetylated. Acetylation is carried out by adding 10 µL of the Acetylation Reagent (as prepared below) for each 200 µL of the standard, sample and Sample Diluent. Immediately vortex each treated standard, sample or Sample Diluent after addition of the Acetylation Reagent and use within 30 minutes of preparation.

Note: Upon Acetylation, all of the standards and samples diluted in the orange Sample Diluent will change to a pale yellow colour.

ACETYLATED ASSAY REAGENT PREPARATION

We recommend that all standards and samples be run in duplicate to allow the end user to accurately determine cAMP concentrations. Ensure that all samples have reached room temperature and have been diluted as appropriate prior to running them in the kit.

Wash Buffer
Dilute Wash Buffer Concentrate 1:20 by adding one part of the concentrate to nineteen parts of deionized water. Once diluted this is stable at room temperature for 3 months.

Cyclic GMP Conjugate Concentrate
The supplied Cyclic GMP Conjugate Concentrate should be diluted 1:20 with the Conjugate Diluent (ie: 125uL Conjugate Concentrate with 2.375 mL of Conjugate Diluent for a total solution volume of 2.5 mL) Once diluted the Cyclic GMP conjugate is stable for one month when stored at 4°C.

Chemiluminescent Substrate
Mix one part of the Substrate Solution A with one part of Substrate Solution B in a brown bottle. Once mixed the substrate is stable for one month when stored at 4°C.

Acetylation Reagent
Working in a fume hood mix one part of Acetic Anhydride with 2 parts of Triethylamine in a glass test tube. Use the following table to help determine the amount of Acetylation Reagent to make.
Use the Acetylation Reagent within 60 minutes of preparation.

	Number of Samples to be Tested		
Reagent	**20**	**40**	**100**
Acetic Anhydride Volume (uL)	200	400	1,000
Triethylamine Volume (uL)	400	800	2,000
Acetylation Reagent Volume (mL)	0.6	1.2	3

Standard Preparation
All standards and samples should be diluted in glass test tubes.

1. Label one glass test tube as Working Stock and six tubes as #1 through #6.
2. Pipet 150 µL of Sample Diluent into the Working Stock tube and 585 µL of Sample Diluent into tube #1.
3. Pipet 300 µL of Sample Diluent into tubes #2 to #6.

4. The Cyclic GMP stock solution contains an organic solvent. Prerinse the pipet tip several times to ensure accurate delivery. Carefully add 10 µL of the cGMP stock solution to the Working Stock tube and vortex completely.
5. Take 15 µL of the cGMP solution from the Working Stock tube and add it to tube #1 and vortex completely.
6. Take 300 µL of the cGMP solution in tube #1 and add it to tube #2 and vortex completely.
7. Repeat the serial dilutions for tubes #3 through #6. The concentration of Cyclic GMP in tubes 1 through 6 will be 1, 0.5, 0.25, 0.125, 0.0625, and 0.0313 pmol/mL.

Reagent	Working Stock	Standard 1	Standard 2	Standard 3	Standard 4	Standard 5	Standard 6
Sample Diluent	150 µl	585 µl	300 µl	300 µl	300 µl	300 µl	300 µl
cGMP Standard Stock Solution	10 µl						
Working Stock		15 µl					
Standard 1			300 µl				
Standard 2				300 µl			
Standard 3					300 µl		
Standard 4						300 µl	
Standard 5							300 µl
Final Concentration (pM/ml)	40	1	0.5	0.25	0.125	0.0625	0.03125

STANDARD AND SAMPLE ACETYLATION

1. Pipet 300 µL of Sample Diluent into a glass tube to act as the Zero standard/NSB tube.
2. Add 15 µL of Acetylation Reagent to this tube and vortex immediately. **Proceed to assay within 30 minutes.**
3. Pipet 200 µL of each standard and sample to be tested into glass tubes.
4. Add 10 µL of the Acetylation Reagent into each tube and vortex immediately. **Proceed to assay within 30 minutes.**

NOTE: Samples and Sample Diluent will turn from orange to pale yellow upon acetylation.

ACETYLATED ASSSAY PROTOCOL

1. Add 50 µL of Plate Primer into all wells used.

Failure To Add Plate Primer To ALL Wells First Will Cause Assay To Fail.

2. Pipet 75 µL acetylated Sample Diluent into the non-specific binding (NSB) wells.

3. Pipet 50 µL of acetylated Sample Diluent into wells to act as maximum binding wells (B0 or 0 pg/mL).

4. Pipet 50 µL of acetylated samples or standards into wells in the plate.

5. Add 25 µL of the diluted cGMP Conjugate to each well using a repeater or multichannel pipet.

6. Add 25 µL of the cGMP Antibody to each well, except the NSB wells, using a repeater or multichannel pipet.

7. Cover the plate with the plate sealer and shake the plate for 15 minutes at room temperature.

8. Place the covered plate in a 4°C refrigerator for 16 hours.

9. The next morning take the plate from the refrigerator and wash the plate 4 times with the wash buffer. Tap the plate dry on absorbent towels.

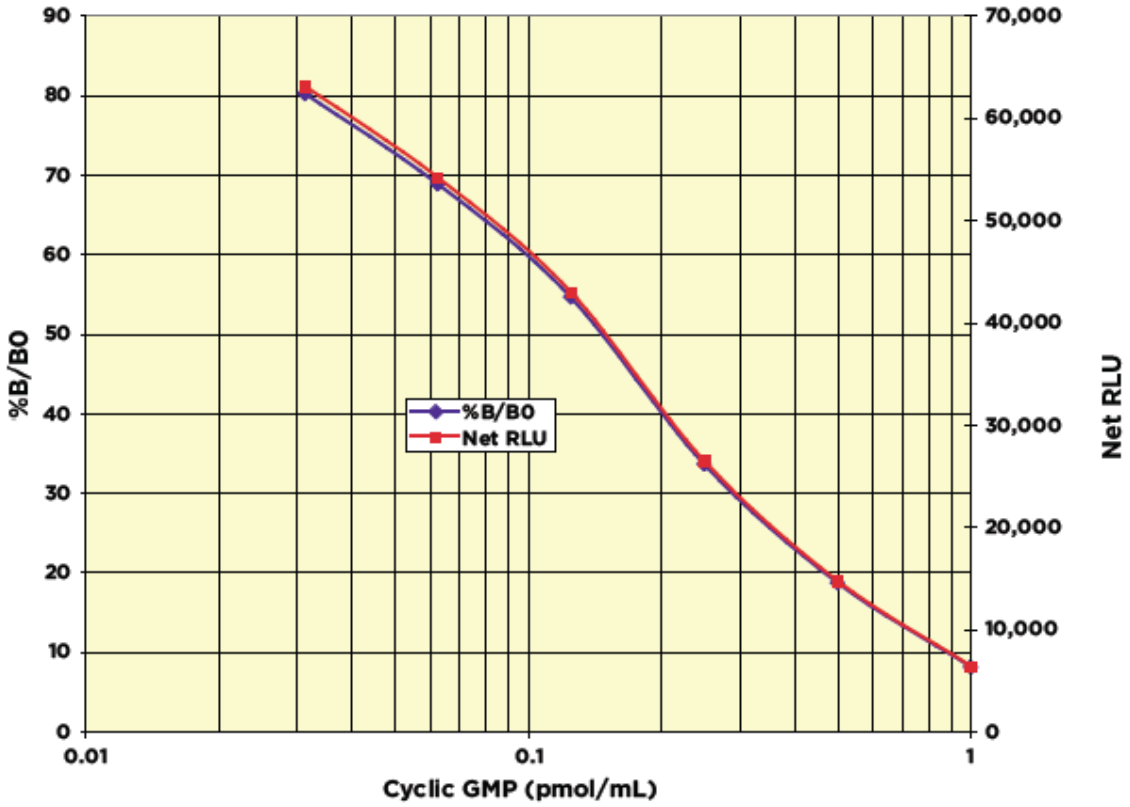
10. Add 100 µL of the mixed Chemiluminescent Substrate to each well, using a repeater or a multichannel pipet.

11. Immediately read the luminescence generated from each well in a mutimode or chemiluminescent plate reader using a 0.1 second read time per well. The chemiluminescent signal will decrease about 40% over 60 minutes.

CALCULATIONS

All luminometers read Relative Light Units (RLU). These RLU readings will vary with make or model of plate reader. Average the duplicate RLU readings for each standard and sample. Create a standard curve by reducing the data using the 4PLC fitting routine on the plate reader, after subtracting the mean RLU's for the NSB. The sample concentrations obtained, calculated from the %B/B0 curve, should be multiplied by the dilution factor to obtain neat sample values.

ACETYLATED FORMAT TYPICAL STANDARD CURVE: EXAMPLE



VALIDATION DATA: ACETYLATED FORMAT

Sensitivity and Limit of Detection - Acetylated
Sensitivity was calculated by comparing the RLU's for twenty wells run for each of the acetylated B0 and standard #6. The sensitivity was determined at two (2) standard deviations from the B0 along the standard curve.
Sensitivity was determined as 0.023 pmol/mL. This is equivalent to 1.15 fmol/well.

The Limit of Detection for the assay was determined in a similar manner by comparing the RLU's for twenty runs for each of acetylated zero standard and a low concentration acetylated human sample.
Limit of Detection was determined as 0.019 pmol/mL. This is equivalent to 0.95 fmol/well.